IO$_4^-$-stimulated crosslinking of catechol-conjugated hydroxyethyl chitosan as a tissue adhesive

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Abstract: Catechol-functionalized polymers are of particular interest because of their strong water-resistant adhesive properties. Hydroxymethyl chitosan (HECTS) has been used as an implantable biomaterial having good water solubility, biodegradability and biocompatibility. Here, hydrocaffeic acid (HCA) grafted HECTS (HCA-g-HECTS) was prepared through carbodiimide coupling and the tethered catechol underwent periodate oxidation and redox chemistry. The gelation time of these cross-linked HCA-g-HECTS hydrogels decreased with increasing molar ratio of cross-linker to catechol, IO$_4^-$-induced cross-linked HCA-g-HECTS hydrogels exhibited much stronger storage modulus and temperature stability than hydrogels made by Fe$^{3+}$-triggered cross-linking. The IO$_4^-$-stimulated HCA-g-HECTS hydrogels were biocompatible on a cellular level when the molar ratio of IO$_4^-$ to catechol group was less than 0.5:1. The hydrogels prepared with a 0.125:1 molar ratio of IO$_4^-$ to catechol group exhibited high adhesion strength of 73.56 kPa against wet rat skin, and a higher adhesion strength than sutures in a rat wound closure model. This biocompatible IO$_4^-$-stimulated HCA-g-HECTS hydrogel may represent a promising new tissue adhesive. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 107B: 582–593, 2019.

Key Words: hydroxyethyl chitosan, catechol group, hydrogel, tissue adhesive


INTRODUCTION

In nature, mussels are found clumped together with each attached to wave washed rocks through its byssus. The expanded plaques at the distal ends of these threads bind to the rock’s foreign surface. The adhesive in these plaques must be rapid setting, strong and tough, or else the mussel will be be dislodged by waves. Biochemical analysis reveals the existence of dozens of proteins within the byssus. Those confined to mussel plaques are mussel foot proteins (mfp) that include mfp-2, mfp-3, mfp-4, mfp-5 and mfp-6.1-5 The secret behind a mussels’ adhesive is a rare amino acid called dihydroxyphenylalamine (DOPA).5 Mfp-3 and mfp-5 in the interfacial region between the plaque and the substratum contain high amount of DOPA.2,3,4,5 Recent research suggests that the reduced catechol form of DOPA binds directly to surfaces, and oxidized states of DOPA, that is, the semiquinone and quinone, regain surface-binding ability after being reduced.7-10 The cohesion with mfps may be brought about through metal ion templating and redox chemistry.11-14

Due to the unique adhesive performance of mfps in wet and turbulent environments, mfps have been the targets for biomimetics.15-17 Typically, polymers are chemically derivatized with catechols in the form of DOPA or DOPA mimetic functional groups, such as 3,4-dihydroxyhydrocinnamic acid (hydrocaffeic acid, HCA) and 3,4-dihydroxybenzaldehyde.18,19 Catechol-conjugated polymers, including poly(ethylene glycols) (PEG–catechol), hyaluronic acid (HA–catechol), alginate (Alg–catechol), heparin (Hep–catechol), dextran (Dex–catechol) and chitosan (Chi–catechol), have recently been reported.20-28

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Chitosan, a polysaccharide obtained by partial deacetylation of naturally occurring chitin, has been used in numerous biomedical applications, including wound dressings, hemostatic materials, drug/gene delivery depots and tissue engineering scaffolds. Various preparation methods, characteristics and biomedical applications of chitosan–catechol have been reported. Chitosan–catechol exhibits excellent solubility at neutral pH solutions and strong adhesiveness to tissue surfaces. HCA has been conjugated to amino groups in the chitosan backbone through carbodiimide coupling. Some chitosan–catechol products have been reported with a degree of catechol conjugation (DoCC) value of 4–30 mol % and even up to 82 mol % for 7-day reactions. Catechol conjugation also enhances the water-solubility of chitosan at a neutral pH, for example, catechol-chitosan with a DoCC of 7.2 mol % has a solubility of ~63 mg/mL at pH 7.0. Reductive amination between the amino groups of chitosan and an aldehyde-terminated catechol represents another approach for the preparation of catechol-chitosan with high DoCC using a short reaction time. The main drawback of catechol-chitosan obtained by reductive amination is that it can dissolve only in acid solutions limiting its application as a tissue adhesive.

In our previous studies, hydroxymethyl chitosan (HECTS) has been demonstrated as an implantable biomaterial with excellent biodegradability, biocompatibility and water solubility over a pH range of 1 to 14. HECTS exhibits good water solubility and dissolution rates between 63% and 99% (50 mg/mL) at pH 2.0. HECTS solutions are advantageous as they can directly replace the acetyl group protons from 1.9 to 1.93 ppm multiplied by 11 because the degree of acetylation of HECTS was determined to be 9%. HECTS hydrogel.

Crosslinking of HCA-g-HECTS by IO

The HCA-g-HECTS was firstly dissolved in distilled water to a concentration of 13 wt %. Then 8, 12, and 16 mg/mL NaIO4 in distilled water was added to 1.0 mL of HCA-g-HECTS solution to make the molar ratio of IO4⁻ to HCA as 0.4:1, 0.7:1 and 1:1, respectively, and mixed to form IO4⁻-stimulated HCA-g-HECTS hydrogel (IO4⁻ - HCA-g-HECTS). Double crosslinking of HCA-g-HECTS was performed as described by Fan et al. with modification. Briefly, genipin was added to HCA-g-HECTS solution and stirred to make a homogeneous solution, followed by the addition NaIO4. The exact preparation conditions for G-IO4⁻-HCA-g-HECTS hydrogel including rheological time-sweep measurement, rheological frequency-sweep measurement, and swelling measurement are provided in Table I.

As comparison, Fe3⁺ triggered HCA-g-HECTS hydrogel (Fe³⁺-HCA-g-HECTS) was performed similarly except for replacing NaIO4 with 6 mg/mL, 8 mg/mL and 12 mg/mL FeCl3 in distilled water. Double crosslinking of Fe³⁺-HCA-g-HECTS by genipin was achieved similarly as G-IO4⁻-HCA-g-HECTS hydrogel.

Rheological measurement

Rheological properties were tested by using a DHR-1 rheometer (TA Instruments, New Castle, DE) equipped with 20 mm diameter stainless steel parallel plate geometry at 25°C and 37°C. The gap between the stainless steel parallel plate geometry and the base plate was 1.0 mm for all tests. Dynamic strain sweep was conducted prior to the frequency sweep, and the strain was determined to be 1% to ensure the rheological measurements were within a linear viscoelastic range. The storage modulus (G') and loss modulus (G'') were determined with frequency sweeps between 0.01 and 100 rad/s. The gelation time was measured by carrying out time sweeps, the frequency and strain was set at 1.0 rad/s and 1%, respectively. The tests were started as soon as the previous report.
as the addition of NaIO₄ (or FeCl₃) solution. Each test was replicated three times.

**Swelling measurement**

IO₄⁻-HCA-g-HECTS hydrogels with the molar ratio of NaIO₄ to HCA to be 0.5:1, 0.25:1, 0.125:1, and 0.0625:1 were prepared by adding NaIO₄ solution (6.5 mg/mL, 100 µL, 50, 25 and 12.5 µL, respectively) into 100 µL of HCA-g-HECTS solution (15 wt %). Double crosslinking of HCA-g-HECTS hydrogels with the molar ratio of NaIO₄ to HCA as 0.0625:1 were formed as described in Table I. All hydrogels were weighed to obtain the initial mass (W₀) and equilibrated mass (W₁) after being immersed in distilled water for 24 h. The swelling percentage was calculated with the Equation (1) and data were expressed as means ± SEM based on 5 tests per sample.

\[
\text{Swelling (\%) = } \left( \frac{W_1 - W_0}{W_0} \right) \times 100\%
\]  

**Wet-resistant adhesion strength measurement**

The adhesive properties of IO₄⁻-stimulated HCA-g-HECTS hydrogels were evaluated by lap shear tests utilizing a SHIMADZU AGS-X 500 N tensile mechanical tester (Kyoto, Japan) as reported with modification. Rat skin samples from sacrificed Sprague-Dawley rats were barbered and cut into 4.0 cm × 1.5 cm rectangular strips. The outside skin then adhered to a PTFE board by cyanoacrylate glue, and immersed in physiological salt solution to ensure it moist before use. HCA-g-HECTS hydrogels (100 µL) were then applied evenly to the inner skin with an area of 1.5 cm × 1.5 cm, where another skin was covered quickly and kept at room temperature for 2 h and 24 h in a highly humid chamber. The adhesion strength was obtained by dividing the maximum load by the corresponding overlapping area. The tests were implemented at a tensile rate of 5 mm/min. Three samples from each hydrogel were tested.

**Cytotoxicity of HCA-g-HECTS and IO₄⁻-stimulated-HCA-g-HECTS hydrogels**

Co-irradiation sterilized HECTS or HCA-g-HECTS (2.0 mg/mL) were dissolved in Dulbecco’s modified eagle’s media (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (100 IU/mL), which was sterilized by 0.2 µm membrane filtration. Mouse fibroblast L929 cells were cultured in DMEM at 37°C in a humidified atmosphere with 5% CO₂. Cells at a density of 1.8 × 10⁴ cells/mL were then seeded in 96-well plates with 200 µL/well, and cultured in the incubator for 24 h. The culture media were then replaced with 200 µL fresh DMEM media, HECTS or HCA-g-HECTS solution, and the plates were then incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 and 48 h, respectively. The viability of cells was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. The cell relative growth ratio (RGR) was calculated as following equation:

\[
\text{RGR (\%) = } \left( \frac{OD_2 - OD_0}{OD_1 - OD_0} \right) \times 100\%
\]  

where OD₀, OD₁ and OD₂ were the average optical density (OD) of the blank, sample (either HECTS or HCA-g-HECTS) and negative control groups, respectively. For each sample 6 wells were used in parallel. Experiments were performed in triplicate.

Following ISO standard 100993-5, the cytotoxicity of IO₄⁻-stimulated-HCA-g-HECTS hydrogels was also evaluated by using extract tests. First, IO₄⁻-stimulated-HCA-g-HECTS hydrogels were formed by addition of different concentrations of sterile NaIO₄ solutions to 100 µL HCA-g-HECTS solution (15 wt %), sterilized by dissolving Co irradiation sterilization HCA-g-HECTS in sterilized distilled water) with the molar ratio of NaIO₄ to HCA to be 0.5:1, 0.25:1, 0.125:1 and 0.0625:1. The hydrogels were then immersed in 8 mL DMEM containing FBS and penicillin/streptomycin for 24 h at 37°C under 100 rpm followed by filtration against 0.2 µm membrane to get the extract solution. L929 cell seeded in 96-well plates was then treated by the extract solution for 24 h and 48 h and evaluated as described above.

**In vivo biocompatibility and biodegradability of IO₄⁻-stimulated-HCA-g-HECTS hydrogels**

The animal experiments in present study were carried out in accordance with the ethical guidelines of the Shandong Province Experimental Animal Management Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twenty adult male Sprague-Dawley rats (weight, 200 ± 10 g) were housed in individual cages and kept under controlled temperature and humidity with free access to food.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>HCA-g-HECTS in Distilled Water (wt %, µL)</th>
<th>0.2% Genipin in 70% Ethanol (µL)</th>
<th>Concentration and Vof NaIO₄ (mg/mL, µL)</th>
<th>Molar Ratio of NaIO₄ to HCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-sweep rheology</td>
<td>13, 100</td>
<td>20</td>
<td>8, 100</td>
<td>0.4:1</td>
</tr>
<tr>
<td>Frequency-sweep rheology</td>
<td>13, 100</td>
<td>20</td>
<td>8, 100</td>
<td>0.4:1</td>
</tr>
<tr>
<td>Swelling</td>
<td>15, 100</td>
<td>20</td>
<td>6.5, 25</td>
<td>0.125:1</td>
</tr>
<tr>
<td>Wet-resistant adhesion</td>
<td>15, 100</td>
<td>20</td>
<td>6.5, 25</td>
<td>0.125:1</td>
</tr>
<tr>
<td>Cytotoxicity and rat skin closure</td>
<td>15, 100</td>
<td>20</td>
<td>6.5, 25</td>
<td>0.125:1</td>
</tr>
</tbody>
</table>

**Table I. Preparation of Double Cross-Linked G-IO₄⁻-HCA-g-HECTS hydrogels**
and water. Four rats were normally raised as a control. Sixteen rats were then anesthetized with intraperitoneal administration of pentobarbital sodium (3% in normal saline) at a dose of 1 mL/kg. The dorsal sides of the rats were gently shaved and disinfected with 75% of alcohol. IO\textsubscript{2}\textsuperscript{-}stimulated-HCA-g-HECTS hydrogel (100 μL, with a molar ratio of NaIO\textsubscript{4} to HCA of 0.125:1), prepared with irradiation sterilized HCA-g-HECTS and filtered through a 0.2 μm membrane was implanted into the dorsal skeletal muscle and the subcutis of eight rats. G-IO\textsubscript{2} hydrogel (four rats) or G-IO\textsubscript{2} g-HECTS hydrogel (100 μL, with a molar ratio of NaIO\textsubscript{4} to HCA of 0.125:1, double cross-linked with 20 μL genipin) was similarly implanted. On the 3rd day and the 7th day after the surgery, four rats of each gel group were sacrificed, the muscles and skin at the injection sites were photographed, collected, fixed in 4% formalin, embedded in paraffin, and after hematoxylin and eosin (H&E) staining the 5 μm thick tissue sections were visualized.

**In vivo wound closure by IO\textsubscript{2} stimulated-HCA-g-HECTS hydrogels**

Based on the *in vitro* lap shear testing, IO\textsubscript{2} stimulated-HCA-g-HECTS hydrogels (with the molar ratio of NaIO\textsubscript{4}/HCA to be 0.125:1) and G-IO\textsubscript{2} g-HECTS hydrogel double cross-linked with genipin were selected for *in vivo* wound closure evaluation. After anesthetization and sterilization of the rat skin, 2 full-thickness wounds (2 cm long × 0.5 cm deep) were made on the dorsum of each Sprague–Dawley rats (female, average weight of 300 ± 50 g). One wound on each rat was closed by dropping 100 μL of sterilized IO\textsubscript{2} -HCA-g-HECTS hydrogel (four rats) or G-IO\textsubscript{2} -HCA-g-HECTS hydrogel (four rats) into the wound followed by waiting for about 2 min. The other wounds were closed by conventional suturing as the control. At two days post-wounding, the test animals were sacrificed and skin tissues at wound sites were excised for histological analyses. These sections were stained with H&E for morphological assessment. The wound closure was evaluated by measuring the tensile mechanical strength of the tissues at the incision site.

**Statistical analysis**

Experimental data in present study was expressed as mean ± standard derivation. Statistical analysis of data was performed by one-way ANOVA with SPSS (VERSION 20.0). The statistical significance was evaluated by Student’s *t* test, with *p* < 0.05 as the minimal level of significance.

**RESULTS**

**Synthesis and characterization of HCA-HECTS conjugates**

Crosslinking primary amines to carboxylic acid groups by using EDC is a powerful and versatile tool for bio-conjugation, which has been used successfully for preparing water-soluble hydrocaffeic acid (HCA) branched chitosan. Based on the fewer unsubstituted NH\textsubscript{2} groups in hydroxyethyl chitosan (HECTS) than found in the original chitosan, EDC/sulfo-NHS was applied for the synthesis of HCA grafted HECTS (HCA-g-HECTS) in this study. The synthetic scheme is illustrated in Figure 1(A). HPLC chromatogram of HCA-g-HECTS shows a symmetric peak in both UV\textsubscript{280nm} and RID detectors, demonstrating that HCA had been conjugated to HECTS [Figure 1(B)]. In comparison with the FTIR spectrum of HECTS, new absorption peaks at 2712 cm\textsuperscript{-1} and 1600 cm\textsuperscript{-1} appeared in the HCA-g-HECTS spectrum due to aromatic and aliphatic C=O stretching, further demonstrating the successful conjugation of HCA to HECTS [Figure 1(C)]. The 1\textsuperscript{H} NMR spectra [Figure 1(D)] exhibit signals at δ 6.6–6.8 ppm arising from the aromatic ring protons of catechol group of HCA- g-HECTS. The degree of HCA conjugation reached to 14.8% using the optimized reaction conditions, a molar ratio of EDC:sulfo-NHS:HCA:HECTS: 3:1:1:5 at pH 5.5 with a reaction time of 3 days.

**IO\textsubscript{2} stimulated HCA-HECTS hydrogels formation**

Cross-linking of HCA-HECTS can be achieved by addition of oxidants, such as sodium periodate. The catechol group can be, thus, oxidized to be highly reactive quinone that is capable of intermolecular cross-linking. To compare periodate cross-linked hydrogels with Fe\textsuperscript{3+} mediated HCA-HECTS hydrogels (Fe\textsuperscript{3+}-HCA-HECTS), which could coordinate theoretically all three catechol groups, molar ratios of 0.4:1, 0.7:1 and 1:1 (IO\textsubscript{2} to catechol groups in HCA-HECTS) were selected to form the NaIO\textsubscript{4} triggered HCA-HECTS hydrogels (IO\textsubscript{4} -HCA-HECTS). The color of the three hydrogels prepared using three different molar ratios of IO\textsubscript{2} to catechol groups were yellow, light brown, and brown, respectively. As shown in Table II, the gelation time of IO\textsubscript{2} -HCA-HECTS began much sooner with the increased molar ratio of IO\textsubscript{2} to catechol group in HCA-HECTS. When the molar ratio of IO\textsubscript{2} to catechol group increased from 0.4:1 to 1:1, the time for gel formation decreased from 281 s to 79 s. By changing the gelation temperature from 25°C to 37°C the gelation occurred more quickly. Thus, the gelation time of IO\textsubscript{2} -HCA-HECTS was dependent on the molar ratio of IO\textsubscript{2} to catechol group and gelation temperature. Genipin is a biocompatible cross-linker that can spontaneously react with the primary amino group of HECTS. Double cross-linking with genipin resulted in a much quicker gelation of HCA-HECTS than did single cross-linking. After adding genipin, the gelation time of IO\textsubscript{2} stimulated HCA-HECTS hydrogel at 37°C was <20 s (Table II).

**Dynamic rheology properties of IO\textsubscript{2} -HCA-HECTS hydrogels**

The viscoelastic properties of HCA-HECTS hydrogels were determined using oscillatory rheometry. The frequency sweep of HCA-HECTS by IO\textsubscript{2} \textsuperscript{-} triggered gelation at different molar ratio of IO\textsubscript{2} to catechol group is illustrated in Figure 2(A). At each molar ratio of IO\textsubscript{2} to catechol group, the formed hydrogel exhibited typical features of a chemically cross-linked elastic polymer with a frequency-independent behavior and a higher storage modulus (G’) than loss modulus (G”). A higher G’ was obtained by increasing the molar ratio of IO\textsubscript{2} to catechol groups. When the molar ratio of IO\textsubscript{2} to catechol groups was 1:1, the G’ reached 60 Pa, a value ten-times higher than that of Fe\textsuperscript{3+}-HCA-HECTS at the same molar ratio of cross-
linker to catechol groups [Figure 2(D)]. As the temperature increased from 25 to 37°C, both G₀ and G₀₀ of IO₂-HCA-g-HECTS showed no obvious change [Figure 2(B)]. These results suggest that IO₂-HCA-g-HECTS is both highly stable and uniform. After double crosslinking of IO₂-HCA-g-HECTS (IO₂ to catechol group was 0.4:1) with genipin, G₀ and G₀₀ were hundreds of times stronger than that of the IO₂ cross-linked hydrogels [Figure 2(C)]. Considering the much shorter gelation time by adding genipin (as listed in Table I), double cross-linking with genipin provides a potentially biocompatible way to decrease the concentration of IO₂-HCA-g-HECTS hydrogel.

The Fe³⁺-HCA-g-HECTS hydrogel shows behavior of both a non-covalent cross-linking and a covalent cross-linked elastic, with both temperature sensitivity and a much lower G' than IO₂-HCA-g-HECTS hydrogels [Figure 2(D–F)]. The double cross-linked genipin (G-Fe³⁺-HCA-g-HECTS) hydrogels had both G' and G₀₀ values significantly higher than those of Fe³⁺-HCA-g-HECTS in a frequency-independent manner [Figure 2(F)].

Swelling of HCA-HECTS hydrogels
The swelling property of a hydrogel is important for both mass transfer and cellular function properties.⁷ The degree of equilibrium swelling for a hydrogel depends on the cross-link and charge densities of its polymer network. The swelling ratios of HCA-HECTS hydrogels were calculated by measuring the change of the weight of hydrogels in distilled water at 37°C. Swelling studies indicated that equilibrium swelling of the hydrogel composites had been reached after being immersed in distilled water overnight (data not shown). The swelling of IO₂-HCA-g-HECTS was studied as a function of the molar ratio of IO₂ to catechol group (Figure 3). The degree of swelling of HCA-g-HECTS hydrogels decreased with an increase of IO₂ to catechol, suggesting an increased cross-linking of the hydrogel. Keeping the molar ratio of IO₂ to catechol as 1:0.0625, double cross-linking with genipin could decrease the swelling of IO₂-HCA-g-HECTS hydrogel.

Water resistant adhesion
The lap shear tensile strength of HCA-g-HECTS and its hydrogels gluing onto wet rat skin is provided in Table III.

### Table II. Gelation Point of HCA-g-HECTS Hydrogels

<table>
<thead>
<tr>
<th>Crosslinker</th>
<th>Cross-Linker to HCA (Molar Ratio)</th>
<th>Gelation Point at 25°C (s)</th>
<th>Gelation Time at 37°C (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IO₂</td>
<td>0.4:1</td>
<td>281</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>0. 7:1</td>
<td>144</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>79</td>
<td>&lt;20</td>
</tr>
<tr>
<td>G/IO₂</td>
<td></td>
<td>21</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

* Double cross-linking of IO₂ stimulated HCA-g-HECTS (molar ratio of IO₂ to HCA was 0.4:1).
The adhesion strength is highly dependent on the overlapping time and the formulation to form the hydrogels. After overlapping for 15 min, IO₄⁻-HCA-g-HECTS, at a molar ratio of IO₄⁻ to catechol group at 0.25:1, slightly improved adhesion strength as compared with that of pure HCA-g-HECTS. There was also no significant difference in adhesion strength among the three IO₄⁻-HCA-g-HECTS hydrogels prepared from the molar ratio of IO₄⁻ to catechol group as 0.125:1, 0.25:1 and 0.5:1. When the overlapping time reached to 24 h, the adhesion strength of both HCA-g-HECTS and IO₄⁻-HCA-g-HECTS clearly increased. The adhesion strength of IO₄⁻-HCA-g-HECTS under the molar ratio of IO₄⁻ to catechol group at 0.125:1 showed the highest value of 73.56 kPa, increasing over 70-times as compared to the hydrogel overlapped for 15 min, suggesting that the conjugation of IO₄⁻-HCA-g-HECTS to skin occurs much more

FIGURE 2. Rheology analysis (frequency sweep) of HCA-HECTS hydrogel. Storage modulus (G') and loss modulus (G'') of IO₄⁻ stimulated HCA-g-HECTS hydrogels formed (A) in different molar ratio of Fe³⁺ or IO₄⁻ to HCA at 25°C, (B) at different gelation temperature (IO₄⁻ to HCA was 1:1), and (C) double cross-linking with genipin (IO₄⁻ to HCA was 0.4:1). For comparison, the rheology analysis of Fe³⁺ triggered HCA-g-HECTS hydrogels were shown in (D–F). Storage modulus (G') and loss modulus (G'') of Fe³⁺ triggered HCA-g-HECTS hydrogels formed (D) in different molar ratio of Fe³⁺ to HCA at 25°C, (E) at different gelation temperature (Fe³⁺ to HCA was 0.4:1), and (F) double cross-linking with genipin as a second cross-linker (Fe³⁺ to HCA was 0.4:1).
slowly than does the formation of hydrogel. As expected, the adhesion strength increased with the decreasing molar ratio of IO\textsubscript{4} to catechol group, since IO\textsubscript{4} was required to form the hydrogel but IO\textsubscript{4} also consumed catechol groups that were responsible for tissue adhesion. It is noteworthy that the adhesion strengths of IO\textsubscript{4}-HCA-g-HECTS, at molar ratios of IO\textsubscript{4} to catechol group as 0.5:1 and 0.25:1, were higher than that of HCA-g-HECTS free from cross-linker. Keeping the molar ratio of IO\textsubscript{4} to catechol group at 0.125:1, double cross-linking IO\textsubscript{4}-HCA-g-HECTS with genipin slightly decreased the adhesion strength.

**Cytotoxicity of HCA-g-HECTS conjugates and HCA-g-HECTS hydrogels**

Quantitative MTT assay was used to evaluate the cytotoxicity of HCTS, HCA-g-HECTS by DMEM cell culture medium on L929 cells after one day and two days of culture. Neither HCTS nor HCA-g-HECTS in the concentration from 0.0625 mg/mL to 2.0 mg/mL inhibited the proliferation of L929 cells, suggesting that they were nontoxic on a cellular level [Figure 4(A,B)].

There are reports on the good biocompatibility of Fe\textsuperscript{3+}, IO\textsubscript{4} and genipin induced cross-linking of catechol group conjugated polymers\textsuperscript{25,42}. The MTT assay of the extract solution from IO\textsubscript{4}-HCA-HETCS hydrogels prepared with different molar ratio of IO\textsubscript{4} to HCA is shown in Figure 4(C). A significant inhibition of cell growth was found when culturing in the extract solution of hydrogel with the molar ratio of IO\textsubscript{4} to HCA as 1:1, while no reduction of cell growth (with a RGR exceeding 100%) appeared upon treatment with the extract solution of hydrogel with a lower molar ratio of IO\textsubscript{4} to HCA. The amount of genipin used for double cross-linking IO\textsubscript{4}-g-HCA-g-HECTS also showed an effect on the cell growth. Since IO\textsubscript{4}-g-HCA-g-HECTS at a molar ratio of IO\textsubscript{4} to HCA of 0.125:1 exhibited the highest adhesion strength on wet rat skin, it was further cross-linked with different volumes of 0.2% genipin. Using the three genipin volumes (10, 15 and 20 μL), hydrogel extracts were found to be noncytotoxic, with a RGR >85% [Figure 4(D)]. Based on these results, IO\textsubscript{4}-HCA-g-HECTS prepared using molar ratio of IO\textsubscript{4} to HCA of 0.125:1 and double cross-linked hydrogel with 20 μL genipin were further evaluated for in vivo biocompatibility.

**In vivo biocompatibility of IO\textsubscript{4} triggered HCA-g-HECTS hydrogels**

The in vivo safety analysis of HCA-g-HECTS hydrogels was obtained by implanting the hydrogels subcutaneously or into the dorsal skeletal muscle. The surgical procedures are presented in Figure 5(E). As shown in Figure 5(A,B), the size of both implanted HCA-g-HECTS hydrogels became smaller on the 3rd day than the initial size of post implantation, no inflammation was observed at the implantation site in skeletal muscle. A red trace in the subcutis around the implanted IO\textsubscript{4}-HCA-g-HECTS hydrogel was observed, however, implying a slight inflammatory reaction. By day 7, the gels were partly adsorbed and tissue necrosis, hyperemia, and hemorrhaging were not observed in either the skeletal muscle or the subcutis [Figure 5(A,B)]. Histological sections of the tissue surrounding the hydrogels were examined 3 and 7 days post implantation [Figure 5(C,D)]. In contrast to normal tissue, marked accumulation of inflammatory cells in the subcutis and muscle was observed at the injection site on day 3 but these progressively decreased and become slight by day 7. Histological response in rats after implantation of HCA-g-HECTS hydrogels was summarized in Table IV based on the method and criteria described by De Jong et al.\textsuperscript{48,49}

### Table III. Lap Shear Adhesion Test Results of HCA-g-HECTS and Its Hydrogels Gluing on Wet Rat Skin After Overlapping for 15 min and 24 h

<table>
<thead>
<tr>
<th>Cross-Linker to HCA (Molar Ratio)</th>
<th>Adhesion Strength (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>HCA-g-HECTS</td>
<td></td>
</tr>
<tr>
<td>IO\textsubscript{4}-HCA-g-HECTS</td>
<td>0.125:1</td>
</tr>
<tr>
<td></td>
<td>0.25:1</td>
</tr>
<tr>
<td></td>
<td>0.5:1</td>
</tr>
<tr>
<td>G-IO\textsubscript{4}-HCA-g-HECTS</td>
<td></td>
</tr>
</tbody>
</table>

\* Double cross linking of IO\textsubscript{4} stimulated HCA-g-HECTS with genipin (molar ratio of IO\textsubscript{4} to HCA was 0.125:1). Data in the table are the mean ± SD.

**In vivo rat skin incision closure**

In the rat skin incision model, the bleeding at the incision site in the rat skin quickly slowed by applying either IO\textsubscript{4}-HCA-g-HECTS or G-IO\textsubscript{4}-HCA-g-HECTS hydrogels. The wound openings closed within 2 min and remained closed during the observation (until 2 days post treatment) [Figure 6(A,B)]. The histological examination of incision area [Figure 6(C)] at day 2 post treatment, with IO\textsubscript{4}-HCA-g-HECTS and
G-IO$_4^-$-HCA-g-HECTS hydrogels, showed lighter infiltrating of inflammatory cells as compared with those closed with sutures. The tensile strength of incision at 2 days post closure with IO$_4^-$-HCA-g-HECTS, G-IO$_4^-$-HCA-g-HECTS hydrogels and sutures is illustrated (Figure 7). Skin treated with both HCA-g-HECTS hydrogels had slightly higher tensile strength than suture-closed skin, and double cross-linked IO$_4^-$-HCA-g-HECTS showed the highest tensile strength of 315.9 ± 66.3 kPa.

DISCUSSION

Polymers were functionalized with various catechol derivatives having strong wet-adhesive properties. Chitosan-catechol reportedly has various biomedical applications. Based on their good water solubility, biodegradability and biocompatibility properties, HECTS and HCA-g-HECTS were prepared in this work and their wet-adhesive properties were studied. Our results showed that HCA-g-HECTS with a HCA conjugation degree of 14.8% exhibited excellent water-solubility (150 mg/mL in water), and good biocompatibility on cellular level even though it reportedly inhibited cell growth by reactive oxygen species released during catechol oxidation.

Polymer hydrogels should adhere to tissue with sufficient mechanical strength to stay intact to effectively act as tissue adhesives. Mussel-inspired, catechol-conjugated polymers can strongly bind to both inorganic and organic surfaces, and self-bond through oxidation or metal ion coordination to form cross-linked hydrogels. The consumption of catechol for cohesion has been found to diminish the catechol adhesion to mica. Therefore, it’s important to optimize gelation conditions while maintaining strong tissue adhesion strength.

Cross-linking of HCA-g-HECTS can be achieved by addition of oxidants such as sodium periodate. When catechol is oxidized to semiquinone or quinone, it becomes highly reactive and can participate in intermolecular covalent cross-linking and interfacial covalent bonding to the various nucleophilic functional groups (that is, –NH$_2$, –SH, imidazole) of biological substrates. The oxidative cross-linking of catechol is dependent on multiple factors including the type of oxidant, the concentration of oxidant and the pH. Peri-odate mediated cross-linking involves the polymerization of...
\( \alpha, \beta \)-dehydro form of the catechol, with a maximum rate of cross-linking occurring for periodate to catechol molar ratios between 1 and 0.5. Additionally, the rate of cross-linking increases with increasing pH, due to an elevated conversion of catechol to quinone at a more basic pH values.\(^{52}\)

Under the neutral conditions, our results showed that IO\(_4^-\) triggered HCA-g-HECTS cross-linking was highly dependent on the molar ratio of IO\(_4^-\) to catechol groups and the cross-linking temperature. As expected, the gelation time decreased obviously with the increasing molar ratio of IO\(_4^-\) to catechol and temperature because of the formation of more quinone and a faster cross-linking. In contrast to the complicated dynamic rheological properties of Fe\(^{3+}\)-HCA-g-HECTS, IO\(_4^-\)-HCA-g-HECTS exhibited typical features of a covalently cross-linked elastic polymer with a frequency-independent behavior and a greater \( G' \) than \( G'' \). The \( G' \) of IO\(_4^-\)-HCA-g-HECTS hydrogel was ten-times higher than that of Fe\(^{3+}\)-HCA-g-HECTS hydrogel, indicating a rapid, efficient and stable cross-linking of the IO\(_4^-\) oxidized catechol groups.

The adhesion strength of IO\(_4^-\) triggered HCA-g-HECTS on wet rat skin increased with a decreasing molar ratio of IO\(_4^-\) to catechol. The highest was obtained when the molar ratio of IO\(_4^-\) to catechol was 0.125:1. In this case, the balance

FIGURE 5. A,B: Microscopic observation of the implant site of 100 \( \mu \)L. (a) IO\(_4^-\)-HCA-g-HECTS hydrogels and (b) G-IO\(_4^-\)-HCA-g-HECTS hydrogels at (1) day 3 and (2) day 7 after implantation in (A) subcutis and (B) skeletal muscle. C,D: Histological examination of the inflammatory reaction and biodegradation of (a) IO\(_4^-\)-HCA-g-HECTS hydrogels and (b) G-IO\(_4^-\)-HCA-g-HECTS hydrogels at (1) day 3 and (2) day 7 after implantation in (C) subcutis and (D) skeletal muscle. (c) Subcutis and (d) skeletal muscle of normal rat as a control. (100×) The molar ratio of IO\(_4^-\) to HCA in IO\(_4^-\)-HCA-g-HECTS hydrogels is as 0.125:1, and 20 \( \mu \)L genipin is added to make G-IO\(_4^-\)-HCA-g-HECTS hydrogels. E: Surgery procedures of implanting the hydrogels into subcutaneously or into the dorsal skeletal muscle.
between catechol group oxidized for cross-linking and tissue adhesion and the un-oxidized catechol for tissue adhesion made the hydrogel the best as a tissue adhesive. Double cross-linking by genipin weakened the adhesion strength of IO$_4^{-}$-HCA-g-HECTS possibly due to the embedding of some catechol groups in HECTS network generated by genipin, which could not contact with skin. In vivo adhesion evaluations showed that genipin double cross-linked IO$_4^{-}$-HCA-g-HECTS exhibited higher adhesion strength than did the mono-cross-linked IO$_4^{-}$-HCA-g-HECTS hydrogel. The difference in adhesion strength measured in vitro and in vivo might come from the different adhesion direction in rat skin and the adhesion time. In vitro adhesion is between the subcutis of two pieces of rat skin, while the in vivo adhesion is between rat dorsum skins, newly cut through by a blade, and the skin edge containing layers of epidermis, dermis and subcutis, and blood vessels. The different interactions between hydrogel and skin may be due to the compositional differences in the skin interface. Adhesion time can also impact the adhesion strength (Table III). The adhesion time of in vitro and in vivo experiments was 24 and 48 h, respectively. This time difference can not be ignored as a factor influencing the in vitro and in vivo adhesion strengths. Interestingly, both HCA-g-HECTS hydrogel and double cross-linked IO$_4^{-}$-HCA-g-HECTS hydrogel exhibit higher tensile strength in rat skin incision closure model than did the suture closure. It was noteworthy that tissue healing is a complex and dynamic process of replacing devitalized and missing cellular structures and tissue layers.$^{53}$ A combination of macroscopic and histological observations, measurements of wound healing markers, often in combination with analyses of cellular and immunologic responses should be done to evaluate the progress of wound repair in the future.

NaIO$_4$ is toxic to cells but polymer-catechol hydrogels, for example, calcium-free alginate-catechol hydrogels formed by oxidation of low concentration of NaIO$_4$ show low cytotoxicity and good biocompatibility.$^{25}$ The cytotoxicity of extract solution of IO$_4^{-}$-HCA-g-HECTS hydrogel was biocompatible when the molar ratio of IO$_4^{-}$ to catechol was lower than 0.5:1. In vivo implantation of IO$_4^{-}$-HCA-g-HECTS hydrogel showed good biocompatibility as the accumulation of inflammatory cells invasion was minimal compared to suture closure.

### TABLE IV. Histological Response in Rats After Implantation of HCA-g-HECTS Hydrogels

<table>
<thead>
<tr>
<th>Implant Implantation Site</th>
<th>IO$_4^{-}$-HCA-g-HECTS Hydrogel</th>
<th>G-IO$_4^{-}$-HCA-g-HECTS Hydrogel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (days)</td>
<td>Subcutis</td>
<td>Skeletal Muscle</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Number of rats examined</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Capsule formation</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Implant degradation</td>
<td>Slight</td>
<td>Marked</td>
</tr>
<tr>
<td>Inflammatory cells invasion</td>
<td>Marked</td>
<td>Marked</td>
</tr>
</tbody>
</table>

### FIGURE 6. Histologic evaluation of rat skin closure. Images of rat’s dorsum skin with created wounds that were closed by IO$_4^{-}$-HCA-g-HECTS hydrogels (i). G-IO$_4^{-}$-HCA-g-HECTS hydrogels (g) and suture (s): (A) 2 min, (B) second day postoperation. Images of hematoxylin and eosin (H&E) staining of sections of wounds at 2 days post-treatment with: (C) Control group (a), IO$_4^{-}$-HCA-g-HECTS hydrogels (b) and G-IO$_4^{-}$-HCA-g-HECTS hydrogels (c). (Original magnification: 100×).
and double cross-linked IO-TECTS hydrogels, G-IO-TECTS hydrogels and suture (control) at 48 h postoperation. 

In conclusion, the present study describes a novel bio-compatible catechol conjugated hydroxethyl chitosan. An IO₂-triggered hydrogel of this polymer is biocompatible at the cellular level and also in tissue. This IO₂-triggered hydrogel with molar ratio of IO₂ to catechol as 0.125:1 exhibits an adhesion strength of 73.6 kPa on wet rat skin. At 2 days post closure, both IO₂-triggered HCA-TECTS and double cross-linked IO₂-HCA-TECTS hydrogels exhibit higher tensile strength in a rat skin incision closure model than do sutures. The IO₂-HCA-TECTS hydrogel represents a promising tissue adhesive and warrants further investigations as an in vivo tissue adhesive.

REFERENCES
